IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Re: Burnie

Application No. 09/214,307

Filed: 4 January 1999

Entitled: Treatment and Diagnosis of Infections of Gram Positive Cocci

DECLARATION

I, James Peter Burnie, hereby declare and state:

1. I am the inventor on the above-captioned US Patent Application. I am familiar with the prosecution and claims including the most recent Office Action dated 28th September 2001.

2. In the aforementioned Office Action, the Examiner has taken the position that certain aspects of the invention are not described sufficiently as to enable one skilled in the art to make use of the invention.

3. The experimental data outlined in Appendix 1, a paper entitled "Identification of an Immunodominant ABC Transporter Homolog in Vancomcyin Resistant *Enterococcus faecium* Infections as a Potential Target for Antibody Therapy" and Appendix 2, were carried out and supervised by me. Appendix 1 shows that polyclonal rabbit antisera was produced against peptides 1 and 2 and human recombinant antibodies was produced against peptides 1 to 4. Epitopes RVAI, KVGIV and FGPKNF correspond to SEQ ID Nos 6, 7 and 8, respectively. An assessment of the protective potential of these antibodies was carried out in a mouse model of the infection. Results obtained in mice using these antibodies are detailed at the "Animal assessment" on page 7 and "Assessment in mice" on page 10 sections and show therapeutic efficacy in mice using antibodies specific against the ABC transporter protein and specific fragments of it.

Appendix 2 demonstrates the utility of antibody fragments, which are freely available and not linked to a phage, against epitopes LKPIRK *KVGIV* FQFP in mice. These experiments were carried out with both vancomycin resistant and sensitive *Enterococcus faecium* strains and with two different inocula (10^8 and 10^9). As is shown in Table 1 of Appendix 2, after administration of the antibody to the animal model, there is a reduction in the bacterial cell counts from the kidney, liver and spleen. Again, this data demonstrates the therapeutic efficacy of an antibody raised to the epitope KVGIV against *Enterococcus faecium* infections.

4. From this experimental data, it can be concluded that use of antibodies specific against the ABC transporter protein and specific fragments thereof will result in a therapeutic effect.

5. The skilled man would reasonably expect that such therapeutic efficacy in mice would also result in a beneficial therapeutic effect in humans. In this field, animal models are used to study the pathogenesis, diagnosis and development of potential treatments for many bacterial infections. It is well known to the skilled man in this field that such efficacy of antibodies in an animal model will be applicable to humans. Animal models are, thus, the starting points for the development of new therapies for diseases and it is reasonable for the skilled man to expect a similar therapeutic effect based on studies in animal models.

6. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true: and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any passing issue thereon.

James Peter Burnie

21 Jan 02, Manchester, UK.

Place & Date

Identification of an Immunodominant ABC Transporter Homolog In Vancomycin Resistant *Enterococcus faecium* Infections as a Potential Target for Antibody Therapy

James P Burnie, T. Carter, G. Rigg, S. Hodgetts, M Donohoe and R. C. Matthews

Abstract

The occurrence of an outbreak of septicemias due to VRE, in Manchester, United Kingdom, provided an opportunity to examine the antibody responses in patients infected by the same strain. Immunoblotting sera from 24 cases, six of whom died, showed an immunodominant cluster of antigens at 34, 54, 57 and 97 kDa, with a statistically significant correlate between survival and immunoglobulin G to the 34 kDa (P=0.0333) and 97 kDa (P=0.0295) bands. An ORF that encodes the 34 kDa antigen (putative VRE ABC1) was identified by sequencing a positive clone obtained by screening a genomic expression library of VRE with seropositive serum and peritoneal dialysate. The derived amino acid sequence showed sequence homology with ABC transporters, with a Walker A and Walker B motif and 57% homology with YbxA from *Bacillus subtilis*. A partial sequence (putative VRE ABC2) was also obtained, in the same recombinant clone, of a second ABC transporter which had a 72% homology with YbaE from *B. subtilis*. Affinity selection with the seropositive serum and peritoneal dialysate used to screen the library, showed that the eluted antibody bound to the 97 kDa band, and direct amino acid sequencing identified this as a possible ABC transporter. Rabbit antiserum against peptides representing Walker A and an area adjacent to the Walker B site crossreacted with bands at 34, 54, 97, 110 kDa and at 30, 34 and 54 kDa respectively. This therefore appeared to be an immunodominant complex of ABC transporters of which the smallest was the 30 kDa antigen. Epitope mapping of this antigen with seropositive patients' sera delineated three linear epitopes (KVGIV, FGPKNF and RVAI). The Walker A site represented by peptide 1 (GHNGSGKSTLAKTIN), epitope RVAI represented by peptides 2 (MRRVAIAGVLAMPRE) and 3 (ELSGGQMRRVAIAGV), epitope KVGIV by peptide 4 (LKPIRKKVGIVFQFP), and recombinant VRE ABC 1 and VRE ABC 2 expressed in *Escherichia coli* pBAD were then used to isolate human genetically recombinant antibodies from a phage antibody display library. An assessment of the protective potential of these antibodies was carried out in a mouse model of the infection. This study suggests that an ABC transporter homolog could be a target for antibody therapy against VRE infections.

INTRODUCTION

Enterococcus faecium accounts for 10-15% of enterococcal isolates, which are the third most common pathogen in hospital patients and responsible for 12% of nosocomial infections, with an associated mortality of 13.7% (27). It is characterised by antibiotic resistance with rates reported of 50% resistant to vancomycin, 52.1% to high level gentamicin, 58.3% to streptomycin and 97% to (31). Vancomycin resistance has been associated with a poor prognosis in patients with bacteremia, survival falling to 24% compared to 59% in cases of bacteremia due to vancomycin sensitive strains (49), although this finding was contested when adjustments were made for the severity of the illness (20).

Hospitals in Northern England have been swept by a strain of vancomycin resistant *Enterococcus faecium* (13) (VRE), which commonly caused septicemia in infected neutropenic and renal patients. Difficulties in treatment, led one affected teaching hospital, in Manchester, to use chloramphenicol as front line therapy in neutropenic patients with a VRE septicemia, with mixed success. These experiences confirmed the lack of clinical response previously reported by others (30) and catalyzed the search for new therapeutic agents.

Before the antibiotic era, antibodies, in the form of immune serum therapy, were widely used to treat a range of bacterial infections (11). In the case of *E. faecalis* and *E. faecium,* complement- dependent neutrophil-mediated killing was enhanced by anti-enterococcal antibodies raised in rabbits or from the sera of patients with endocarditis (1, 2, 23). Phagocytosis-resistant strains of *E. faecium* were identified (2, 41). This resistance was mediated by bacterial carbohydrate and reversed in the presence of complement by a polyclonal rabbit antiserum or derived antibody fragments against one of these strains. (41). The potential of capsular polysaccharides as targets for opsonophagocytic antibodies and as a vaccine candidate have also been reported (24, 25).

Recent developments in antibody engineering have included the use of immunoglobulin mRNA from the antibody-secreting cells of patients who have recovered from a specific infection to produce phage antibody display libraries (9, 10, 35). The cDNA from the immunoglobulin genes encoding heavy and light-chain variable domains are linked together to produce a library of human genetically recombinant antibodies. The retention of the antigen binding capacity of these antibody fragments allows them to be matched with specific antigens or their epitopes. The aim then becomes the identification of antigens which are associated with protective antibodies. This study describes the identification of an ABC transporter homolog as an immunodominant VRE antigen, associated with antibodies with protective potential.

First identified by immunblotting sera from septicemic patients involved in the VRE outbreak, the antigen was sequenced and epitope mapped and used to isolate antibodies which were then assessed in an mouse model of the infection.

MATERIALS AND METHODS

Immunoblotting. An antigen preparation was made from a clinical isolate of VRE grown in Brain Heart Infusion Broth (Oxoid, Basingstoke, UK) at 37^{0}C and fragmented as previously described (8). The VRE was identified by Gram stain and biochemical profile, and confirmed as the endogenous hospital outbreak strain (13) by gel pattern on Pulsed Field Gel Electrophoresis following *Sma*I digestion. It was resistant to ampicillin, vancomycin, teicoplanin, gentamicin. The following sera were examined by immunblotting.

Group 1: Control sera. Sera from hospital inpatients with no evidence of infection or colonisation by VRE (n=60).

Group 2: Faecal carriers. All had either leukaemia (n=29) or chronic renal failure (n=5). Despite faecal carriage, there was no evidence of related sepsis as judged by pyrexia or positive urine, wound or blood culture.

Group 3: Septicemias (survivors). Sera from clinically septicemic patients, who had leukemia and were neutropenic, with blood-culture confirmed VRE, successfully treated by an antibiotic combination including chloramphenicol (n=18). Patients had either leukaemia (n=12) or chronic renal failure with concomitant abdominal sepsis (n=6). In all cases, sera were examined within 72 hours after starting therapy. In nine cases, additional sera were available, taken before the first positive blood culture.

Group 4: Septicaemias (fatal). Sera from patients, who had leukemia and were neutropenic, who died from a VRE septicemia and had at least one positive blood culture within 72 hours of death (n=6). Sera were collected within 72 hours of starting therapy.

Patients' sera were examined at a dilution of 1:10 against immunoblots of VRE, as described previously (8). IgM and IgG were assayed separately, except for the control Group 1 where they were combined. Blots were analysed as described previously (9, 10): antibody was recorded as present if the intensity of the bound antibody was >50 mm by reflectance densitometry (Chromoscan 3; Joyce Loebl); where multiple sequential sera were tested, titers were reported as constant if the variation in height of the trace remained within 5mm whereas a rising antibody response was recorded if there was an increase of at least 30mm.

Identification of antigens from a VRE genomic library. Chromosomal DNA from the clinical isolate of VRE was partially digested by *Sau*IIIa and fragments in the size range 2 to 6 kbp were inserted into the expression vector lambda ZAP Express (Stratagene Ltd., Cambridge, UK) essentially as described by Young and Davies (57). The library was

screened with a serum and peritoneal dialysate fluid (1:100 IgG) from a patient on chronic ambulatory peritoneal dialysis who survived the infection, having had positive blood and peritoneal cultures. The peritoneal dialysate contained antibodies dominant for the bands at 54 and 97 kDa (Figure 1, lane 1) and the serum contained IgG for the bands at 34 and 97 kDa (Figure 2, lane 7). Positive clones were detected by alkaline phosphatase-conjugated goat anti-human immunoglobulin (IgG) (1:5000; Sigma, Poole, UK). Antibodies in the screening serum and peritoneal dialysate (1:10) were affinity purified against positive recombinant plaques. Bound antibody was then eluted with glycine buffer pH 2.8 and screened against an immunoblot of VRE. Direct aminoacid sequencing was performed on the 34 and 97 kDa bands by the Biochemistry Department, University of Cambridge, Cambridge, United Kingdom.

For DNA sequencing the positive clones (Sequenase version 2.0 kit; United States Biochemical, Cambridge, UK), the first set of annealing reactions was done with universal primers, T3 and T7; subsequent primers were derived from the sequences obtained from both the coding and non-coding strands. Homologous proteins to the VRE ABC transporters were identified using a Blast search on the National Center for Biotechnology Information, Baylor College of Medicine, BLAST WWW Server, BCM search launcher, by the BLAST P + BEAUTY program.

Contruction of fusion proteins. Gene fusion techniques were used to confirm the immunogenicity of VRE ABC1 and VRE ABC2. The PCR primers for VRE ABC1 were 5′ GGA GTA ATC ATG GAG CC and 5' CGG ATG TCC ATA ACC AAT CCA CC and for VRE ABC2 5' GGT TAT GGA CAT CCG and 5' GAT CAA GTC CTG CCG TTG G. DNA from positive clones was amplified and the resulting products were ligated into the expression vector Pin Point Xa1 T-Vector to produce a fusion of the antigen with the 13-kDa biotinylation tag sequence of the vector (Promega, Madison, USA). The ligation mixture was used to transform *E.coli* JM109. Potential clones were examined by restriction analysis to confirm the presence of an insert of the correct size. Clones containing inserts of the correct size were grown to early log phase in Luria-Bertani broth and induced with 1mM IPTG and growth continued for 2h. *E.coli* cells were collected by centrifugation and examined for the expression of biotinylated fusion proteins by immunoblot analysis with streptavidin conjugated alkaline phosphatase. Colonies expressing the biotinylated fusion proteins were probed with a negative control serum, a late serum from a septicemic surviving patient (as illustrated in Figure 2, lane 4), the peritoneal dialysate from an infected patient (Figure 1, lane 1) and the serum originally used to screen the VRE library (Figure 2, lane 7). Both VRE ABC 1 and VRE ABC 2 were also cloned into the vector pBAD by means of a pBAD-TA-TOPO cloning kit (Invitrogen Corp., Oxon, United Kingdom), protein induction initiated by 0.02% arabinose and the V5 tagged recombinant protein confirmed by probing with a monoclonal antibody to the V5 tag (1:5,000).

Epitope mapping. This procedure, described by Geyson *et al* (21), was applied to the aminoacid sequence derived for VRE ABC 2. It was synthesised as a series of overlapping nonapeptides on polyethylene pins using an epitope scanning kit (Cambridge Research Biochemicals, Cambridge, United Kingdom), the first nonapeptide consisting of residues 1 to 9, the second residues 2 to 10 etc. An IgG ELISA reaction (A_{405}) was used to determine the reactivity of each peptide with patients' sera (1:200) after 30 min incubation. Sera were examined from both survivors (n=3) and fatal cases (within 72 hours of death; n=3) of septicemia due to VRE, as well as hospitalised control patient who had no evidence of VRE colonisation or infection (n=2) (Table 1).

Rabbit Antisera. Polyclonal rabbit antisera were produced against two synthetic peptides in the animal facility at Manchester University: GHNGSGKSTLAKTIN (peptide 1) representing the Walker A site and MRRVAIAGVLAMPRE (peptide 2) an area before the Walker B site incorporating the epitope RVAI. Each peptide was dissolved in deionized water at a concentration of 2mg/ml and diluted 1:1 with Freund's complete adjuvant (Sigma) before injection into New Zealand white rabbits. Animals were boosted every 2 weeks with the peptide at a concentration of 1mg/ml in Freund's incomplete adjuvant (Sigma). Rabbits were bled after four immunizations and the sera purified by centrifugation. Preimmunization sera and sera from the sequential bleeds were immunoblotted at 1 in 40 against the VRE extract.

Preparation of human genetically recombinant antibodies. A phage antibody display library, derived from a patient who had recovered from *E. faecium* sepsis, was panned as previously described (9, 10, 35), enriching for antigen-specific recombinant antibodies against peptides 1 and 2, ELSGGQ MRRVAIAGV (peptide 3) and LKPIR KKVGIV FQFP (peptide 4) by panning against immunotubes coated with peptide (10 mg/ml). To obtain phages expressing recombinant antibodies against recombinant VRE ABC 1 and ABC 2, the latter were run on SDS PAGE gels, immunoblotted and overlaid with the phage library diluted 1 in 2 in 2% skimmed milk. Bound antibodies were eluted by triethylamine (14 µl/ml water) neutralised by 500 µl of 1M Tris HCl pH7.4 and rescued by log-phase *E. coli* TG1.

Animal Assessment. The VRE was grown overnight at 37°C in Brain Heart Infusion Broth, washed in saline, and the concentration (determined by hemocytometer and plating of dilutions on blood agar) adjusted so that 1×10^9 CFU were injected as a 0.1 ml bolus into the lateral tail vein of female CD1 mice (22 to 24g; Charles River). Two hours after challenge, randomized groups of 10 animals were given intravenously 200 µl of phage types 1-6 (peptide 2), 7 (peptide 3), 8-11 (peptide 4), 12-14 (VRE ABC 1) and 15 and 16 (VRE ABC 2) or a negative control phage (expressing antibody which does not bind *E. faecium*). The phage dosage was limited to $\leq 5 \times 10^8$ phage to avoid toxicity due to the phage

itself. Bacterial cell counts were determined from the kidney, liver and spleen, expressed as the mean $\log_{10}$ CFU per gram plus standard deviation, and the treatment and negative control group counts were then compared using the Mann-Whitney U test. For all tests, a *P* value of <0.05 was considered significant.

RESULTS

Immunblotting Patient Sera. Immunoblotting sera against VRE showed bands of apparent molecular mass ranging from 24 to 150 kDa (Table 2). In patients who recovered from a VRE septicemia (Group 3), bands at 34, 54, 57 and 97 kDa were recognised by $\geq 50\%$. In patients with VRE faecal carriage (Group 2), the 97 kDa was immunodominant, 21 (61%) having IgG and 12 (35%) having IgM to this band, followed by the band at 34 kDa, 15 (44%) having IgG and 8 (24%) having IgM. In contrast only 2 of the 6 fatal cases of VRE septicemia (Group 4) had IgG to the 97 kDa band (33%) whilst none had an antibody to the 34kDa band.

The increased frequency of IgG to the 34 and 97 kDa bands in survivors of VRE septicemia (Group 3) was statistically significant compared to the control group (Group1) ($P < 0.0001$ in each case) and fatal cases (Group 4) ($P = 0.0333$ and $P = 0.0295$ respectively). The increased frequency of IgG against the 54 kDa band in survivors (Group 3) was also statistically significant compared to controls (Group1) ($P< 0.0001$) and faecal carriers (Group 2) ($P = 0.0087$). The increased frequency of IgM to the 54 band in survivors of septicemia (Group 3) was statistically significant compared to controls (Group 1) ($P < 0.001$) and faecal carriers (Group 2) ($P = 0.0022$). The increased frequency of IgM to the 97 kDa band in survivors of septicemia (Group 3) was statistically significant compared to controls (Group 1) ($P = 0.0187$) (Fisher's exact two tailed test; $P \leq 0.05$).

Serial sera were available from 9 patients who survived the VRE septicemia and the majority of these showed rising antibody levels (IgG and/or IgM) to the bands at 34kDa (7 patients), 54kDa (7 patients) and 97 kDa (all 9 patients), as illustrated in Figure 2.

Identification of VRE Antigens. Screening the VRE genomic library with the peritoneal dialysate from a patient infected with VRE, gave a positive clone. Affinity selection, either with the original serum (Figure 2, lane 7) or the corresponding peritoneal dialysate (Figure 1, lane1), demonstrated that when the bound antibody was eluted it cross-reacted strongly with the 97 kDa band and weakly with the bands at 54, 34 and 30 kDa on immunoblot (Figure 1, lane 2). The derived amino acid sequence produced two contiguous open reading frames with both proteins containing ATP-binding domains and sequences homologous to the ABC transporter proteins (18, 32). One of these proteins (putative VRE ABC1) was a complete sequence (apparent molecular weight 30.6kDa) whilst the other coded only for

the amino terminal end of putative VRE ABC2 (apparent molecular weight of fragment 19 kDa) (Figure 3). The consensus ribosomal binding site of *B. subtilis* is AAA GG AGG, which is normally distributed between positions -5 and –11. In the case of the present clone these areas were represented by GAA GG AGT (VRE ABC1) and AAA GG ATG (VRE ABC2). The putative VRE ABC1 ends with a lysine, which in *B. subtilis* is perceived as a common terminal amino acid (42).

The homologies for the genes encoding the putative VRE ABC1 and VRE ABC2 are given in Table 3. The closest database match for the gene encoding VRE ABC1 was *ybxA* (encoding a protein of apparent molecular weight 31.3kDa) and for VRE ABC2 it was *ybaE* (encoding a protein of apparent molecular weight 30.4kDa). In *B. subtilis*, the *ybxA* and *ybaE* genes belong to the same operon, which also includes a gene coding for a membrane spanning domain protein YbaF. These belong to the extruder sub-family 12 group of proteins (40). The VRE genes encoding the putative ABC1 and ABC2 were also homologous to *ykoD*, a gene from *B. subtilis* encoding an ABC protein with an apparent molecular weight of 54.6 kDa. The remainder of the clone contained the carboxy end homologue of ribosomal protein L17 which is the next protein in the genomes of *B. subtilis*, *B. stearothermophilus, B. halodurans* and *M. capricolum.* (5, 6, 29, 52). A search of the unfinished database of *Enterococcus faecalis* V583 found a contig 10502 (TIGR database) which contained a homologue similar to VRE ABC1 (79.2% identity over 279 amino acids, with apparent molecular weight of 30.71 kDa) and a homologue similar to VRE ABC2 (51.9% identity over 289 amino acids, with apparent molecular weight of 32.35 kDa).

Gene fusion constructs. Expression of VRE ABC1 gave a band at 44 kDa, corresponding to a biotinylated recombinant protein of 31 kDa, the biotinylation tag being 13 kDa. Expression of VRE ABC2 gave a band at 32 kDa, corresponding to the tag plus a 19 kDa protein. Both constructs were positive with: the late serum from a surviving septicemic patient; the peritoneal dialysate from an infected patient; and the serum originally used to screen the VRE library. Each was negative with serum from the negative control group. The nonbiotinylated, V5-tagged, arabinose-induced recombinant VRE ABC 1 produced a single band at 31 kDa and VRE ABC 2 a band at 22 kDa when probed with the V5 monoclonal antibody.

Epitope mapping. Epitope mapping identified three sites at which sera from patients who survived VRE septicemia produced a mean optical density (OD), over three or more consecutive wells, at least 2 standard deviations above that of sera from septicemic patients who died or uninfected hospital inpatient controls (Table 1). A comparison with the derived sequences from ybaE demonstrated that each epitope was highly conserved except FGPKNF which is

represented by FGPKMF in *B. subtilis*. Comparison with VRE ABC 1 demonstrated that RVAI was the only conserved epitope and this was used in peptides 2 and 3 for panning.

Antigen Specificity of Rabbit Antisera. Rabbit immunization with peptide 1 gave rise to antiserum which cross-reacted with bands at 34, 54, 97 and 110 kDa (Figure 1, lanes 3 and 4), while immunization with peptide 2 gave antiserum recognizing bands at 30, 34 and 54 kDa (Figure 1, lanes, 5, 6, and 7). Direct amino acid sequencing of the 34 kDa band gave MEP (the amino end of VRE ABC 1), while direct amino acid sequencing of the 97 kDa band gave REFSLQNT, showing an 87.5% identity with an area from Mdr50, a Drosophila P-glycoprotein/multidrug resistance gene homolog which is a member of the ABC superfamily (22).

Human recombinant antibodies. *Bst*N1 fingerprinting of the PCR-amplified recombinant antibody inserts showed that before panning, the library was highly heterogeneous. After four rounds of panning: no focusing occurred against peptide 1; peptide 2 produced multiple copies of 6 types (DNA fingerprint types 1-6); peptide 3 focused a single type (type 7); peptide 4, four types (types 8-11); VRE ABC1 four types (types 12-14) and VRE ABC2, two types (types 15 and 16). Each of these were tested for activity *in vivo*.

Assessment in mice. Phage antibody activity was assessed in terms of a statistically significant reduction in organ colony counts (kidney, liver and spleen) compared to the negative control phage. Activity was greatest with certain phage types against peptide 2 and 4 and least with phages panned against peptide 3 (Table 3). Phage type 6 (against peptide 2) showed a statistically significant reduction in colony counts in all three organs in four independently conducted sets of experiments ($P < 0.05$). The mean reduction in organ colony counts, expressed as $\log_{10}$ CFU per gram, for this phage type 6 were 0.8 – 1.2 (spleen), 0.8 – 1.4 (kidney) and 0.6 – 1.3 (liver) compared to the negative control phage. Phage types 9 and 10 (both against peptide 4) showed a significant reduction in all 3 organs; phage type 14 (against recombinant protein VRE ABC 1) showed a significant reduction in two organs (spleen and spleen); phage types 12 and 13 (against recombinant protein VRE ABC 1) and phage type 15 and 16 (against recombinant protein VRE ABC 2) showed a significant reduction in one organ (spleen). All other phage antibodies, types 1-5 (panned against peptide 2), type 7 (peptide 3) and types 8 and 11 (peptide 4) showed no significant activity.

The scFv against peptide 3 showed little or no activity whereas the scFvs against peptides 2 and 4 and VRE ABC 1 and VRE ABC 2 all showed semilogarithmic or more reductions in one or more organs. Type 6 was the most active producing semilogarithmic or greater reductions in all three organs in four separate experiments.

DISCUSSION

This study characterized the antibody response to VRE septicemia, and identified a cluster of immunodominant putative ABC transporter proteins, which could be targets for potential antibody therapy. The antibody profile in survivors involved in an outbreak of VRE septicemias was compared with that of fatal cases, faecal carriers of VRE and uninfected hospital inpatients. There was a statistically significant increase in the frequency of IgG to the 34 and 97 kDa bands in survivors compared to fatal cases of VRE septicemia and uninfected controls, and in the frequency of IgG and IgM against the 54 kDa band in survivors, compared to uninfected controls and faecal carriers of VRE. Serial sera from 9 patients who survived the VRE septicemia showed rising antibody levels (IgG and/or IgM) to the bands at 34kDa (7 patients), 54kDa (7 patients) and 97 kDa (all 9 patients). Immunodominant bands at 54 and 98 kDa have previously been reported in enterococcal infections (8, 51).

Screening a VRE genomic library with serum and peritoneal dialysate from a patient who survived VRE septicemia and peritonitis and had antibody to the 34, 54 and 97 kDa bands, revealed a positive clone which, on DNA sequencing, comprised two contiguous open reading frames, both encoding putative ABC transporter proteins (VRE ABC1 and VRE ABC2). Members of the ABC (ATP-binding cassette) superfamily share a highly conserved protein, called the ABC module, that not only displays the Walker A and Walker B motifs common to all ATP-requiring proteins, but in addition, a short, highly conserved sequence (consensus LSGGQ) called the signature sequence or linker peptide, which is displayed uniquely by and thus identifies members of the ABC superfamily (45, 46, 56). Both VRE ABC1 and VRE ABC2 had Walker A and Walker B sites and the signature sequence LSGGQ. Immunoblot analysis of the antibody eluted from this positive clone showed crossreactivity with the 97, 54, 34 and 30 kDa bands. Rabbit immunization with synthetic peptides representing the Walker A site and a conserved epitope, RVAI, occurring just before the Walker B site, gave rise to antisera which cross-reacted with bands at 34, 54, 97 and 110 kDa (peptide 1), and bands at 30, 34 and 54 kDa (peptide 2). Direct amino acid sequencing of the 34 and 97 kDa bands also indicated homology with ABC transporter proteins (with VRE ABC1 and a drosophila ABC transporter protein respectively). It seems likely that the 34, 54 and 97 kDa bands associated with antibody responses in patients who recovered from VRE septicemia, are part of an ABC transporter family in VRE.

ABC transporters have also been identified as immunodominant antigens in infections due to *E. faecalis* (15, 53) and Methicillin-Resistant *Staphylococcus aureus* (10). In the latter, recovery from MRSA septicemia was associated with seroconversion of surviving patients to an ABC transporter of 61 kDa (EMRSA-15 ABC protein) (10). Multiple ABC transporters have been implicated as virulence factors in gram positive bacterial infections, pathogenesis being studied

by in vivo expression and signature-tagged mutagenesis (16, 33, 36, 38). Homolgy studies (Table 3) show the closest database match for the gene encoding VRE ABC1 was *ybxA* and for VRE ABC2, *ybaE.*, both encoding monomeric ATPases in *B. subtilis* of apparent molecular weight 31.3kDa and 30.4kDa respectively. The *ybxA* and *ybaE* genes belong to the same operon in *B. subtilis,* which also includes a gene coding for a membrane spanning domain protein YbaF. These are members of the subfamily 12 extruders, which have not been reported in *E. coli* (40). It includes the product of *ykoD* (a dimeric ATPase with an apparent molecular weight of 54.6 kDa) in which the N and C terminal nucleotide binding domains are related to *ybxA* and *ybaE* respectively. The VRE genes encoding the putative ABC1 and ABC2 were also homologous to *ykoD.* The *ybxA* gene is located upstream of the *ybaE* gene in the same operon, which has led to speculation that *ykoD* occurred by fusion of these genes after duplication of the operon. Nevertheless, the presence of an equivalent structure in the archaeon *Methanobacterium thermoautotrophicum* suggests that the fusion occurred early in evolution (48).

Recently MsrC, a member of the ABC transporter family, has been described from *E. faecium* (39, 47). This dimeric ATPase, associated with macrolide resistance, has an apparent molecular weight of 56 kDa and is homologous to MsrA from *S. aureus* (39, 47) which itself is homologous to the EMRSA-15 ABC protein (10). Therapeutic activity has been demonstrated by human recombinant antibodies against this latter molecule in a mouse model of infection (10). The synthetic peptide (peptide 1) representing the Walker A site derived from VRE ABC2 was identical in 9 out of 10 amino acids to the corresponding area in the previously described *E. faecium* MsrC (39, 47), so that an antiserum raised against this peptide would be likely to crossreact with MsrC. The antiserum produced a band on the VRE immunoblot at 54 kDa, suggesting that this might be a homolog of *E. faecium* MsrC. An alternative explanation is that this 54 kDa band is a homolog of YkoD, which has an apparent molecular weight of 54.6 kDa. Another ATPase of 54 kDa has been cloned from *E. faecium* with a different sequence; this was shown to be present in cell well preparations (26).

The function of the putative VRE ABC1 and VRE ABC2 proteins is at this stage unknown. There is some homology (table 4) with proteins involved in cobalt transport (7, 28, 43, 44). The latter in *B. subtilis* has been linked to tetracycline transport (14) whilst *E. faecium* possesses endogenous efflux pumps that excrete fluoroquinolones and chloramphenicol and sometimes tetracycline (34). Cobalt has been shown to activate an aminopeptidase in *Streptococcus sanguis* and *Saccharomyces cerevisiae* (12). Both VRE ABC1 and VRE ABC2 had Walker A, Walker B and the linear peptide LSGGQ which have all been shown to play an essential role in the transport process (46). The VRE ABC1 sequence also demonstrated a short motif at the end of the β5 strand which is thought to be implicated in signal transduction to the ABC domain by sensing conformational changes in the membrane spanning domain upon

substrate binding (45). This switch region is marked in Figure 3 and is centered around an invariant histidine and associated threonine.

Epitope mapping identified three sites at which sera from patients who survived VRE septicemia were significantly more reactive than sera from septicemic patients who died or uninfected hospital inpatient controls. Two of these (KVGIV and RVAI) were successfully incorporated into synthetic peptides, peptide 4 (KVGIV) and peptides 2 and 3 (RVAI). The latter epitope was conserved between VRE ABC1 and VRE ABC2. Therapeutic activity of phages panned against these epitopes and the recombinant VRE ABC1 and VRE ABC2, was assessed in a mouse model of systemic VRE infection. Activity was greatest with phages panned against peptide 2 and 4 and least with phages panned against peptide 3. Phage type 6 (against peptide 2) showed a statistically significant reduction in colony counts in all three organs in four independently conducted sets of experiments ($P < 0.05$). Phage types 9 and 10 (both against peptide 4) showed a significant reduction in all 3 organs; phage type 14 (against recombinant protein VRE ABC1) showed a significant reduction in two organs (spleen and spleen); phage types 12 and 13 (against recombinant protein VRE ABC1) and phage type 15 and 16 (against recombinant protein VRE ABC2) showed a significant reduction in one organ (spleen). All other phage antibodies, types 1-5 (panned against peptide 2), type 7 (peptide 3) and types 8 and 11 (peptide 4) showed no significant activity.

The most therapeutically active antibody (type 6) was produced against synthetic peptide 2 which incorporated the immunodominant epitope RVAI. This sequence is part of the linker peptide between LSGGQ and the Walker B site (45) (Figure 3). The ABC transporter family comprises membrane associated export and import systems which participate in the import and secretion from the cells of many different molecules (sugars, amino acids, oligopeptides, carbohydrates, toxins, ions, drugs etc) (18, 32, 40). In *E. faecium* systems for enterocin A excretion (37) and antibiotic resistance (39, 47) have been described. A humoral response which blocks this process by targeting shared epitopes common to multiple transporters would simultaneously interfere with numerous processes key to the survival of the bacteria and its ability to function as a pathogen. It would explain the widespread immunodominance of these molecules in Gram positive infection (10, 15, 35, 53) and act as a potent method for a single antibody to produce a multitude of therapeutic effects in a diversity of microbes.

Many clinical isolates of *E. faecium* are resistant to neutrophil mediated phagocytosis and killing in the presence of normal human serum. Normal serum would be deficient in antibody against the ABC transporter complex. Immune rabbit serum generated against formalin killed *E. faecium* TX 0016, a phagocytosis resistant strain, reversed this phenomenon for this strain (41). This effect was mediated by the antibody or its fragments and was attributed to

antibodies against the bacterial carbohydrate (54). Polysaccharide biosynthesis in *E. faecalis* involves a cluster of genes containing ABC transporters which suggests that a similar situation occurs in *E. faecium* (54). Antisera raised against formalin (41) or gentamicin (24) killed *E. faecium* is likely to contain antibody against ABC transporter molecules which might, in part, explain its activity. This would be consistent with the results shown by the scFvs in the current work. The potential therapeutic activity of such antibodies is under further investigation, starting with the expression of scFvs in *E. coli* in a phage free form in order to facilitate further assessment with greater antibody concentrations.

Table 1: Epitope map showing sites at which the mean OD value in survivors was at least 2 standard deviations ab ve the mean OD value for fatal cases or uninfected hospital inpatient controls.

Well No.	Epitope sequence	Hospital inpatient controls (n=2)	Septicemic patients who died (n=3)	Septicemic patients who survived (n=3)
50	KVGIV	0.259 ± 0.206	0.259 ± 0.038	0.674 ± 0.393
51	KVGIV	0.279 ± 0.228	0.278 ± 0.028	0.775 ± 0.345
52	KVGIV	0.250 ± 0.182	0.264 ± 0.019	0.551 ± 0.264
53	KVGIV	0.285 ± 0.189	0.356 ± 0.037	0.732 ± 0.434
54	KVGIV	0.342 ± 0.235	0.440 ± 0.034	0.902 ± 0.550
74	FGPKNF	0.445 ± 0.108	0.392 ± 0.028	0.717 ± 0.425
75	FGPKNF	0.321 ± 0.288	0.356 ± 0.07	0.809 ± 0.543
76	FGPKNF	0.274 ± 0.231	0.279 ± 0.048	0.676 ± 0.411
77	FGPKNF	0.334 ± 0.309	0.383 ± 0.042	0.807 ± 0.499
116	RVAI	0.296 ± 0.244	0.319 ± 0.075	0.694 ± 0.444
117	RVAI	0.384 ± 0.347	0.492 ± 0.106	0.837 ± 0.528
118	RVAI	0.444 ± 0.227	0.541 ± 0.062	1.053 ± 0.582
119	RVAI	0.365 ± 0.113	0.419 ± 0.015	0.839 ± 0.407
120	RVAI	0.389 ± 0.192	0.429 ± 0.048	0.847 ± 0.426
121	RVAI	0.365 ± 0.317	0.472 ± 0.073	0.771 ± 0.421

The overlapping amino acid sequences were derived by a comparison of first and last peptide sequences and were used to define the epitopes.

Table 2: Immunoblot analysis of patients' sera against VRE

Antigens (apparent molecular mass; kDa)	No of patients with indicated antibody						
	Controls (Group 1)	Faecal carriers (Group 2)		VRE septicemias: survivors (Group 3)		VRE septicemias: fatal cases (Group 4)	
	(n=60)	(n=34)		(n=18)		(n=6)	
	IgM and IgG	IgM	IgG	IgM	IgG	IgM	IgG
150	0	0	0	1	3	0	1
130	0	0	0	1	1	0	0
110	0	1	5	1	4	0	1
97	3	12	21	5	16	0	2
89	0	1	4	1	5	0	1
87	0	0	0	1	1	0	0
85	2	1	1	1	1	0	0
83	0	0	0	2	1	0	0
81	4	4	3	4	3	0	0
76	3	0	1	0	1	0	0
73	0	0	0	1	1	0	0
71	0	0	1	2	2	0	0
67	2	1	4	4	3	0	0
65	0	0	0	0	2	0	0
59	0	0	0	1	1	0	0
57	7	4	10	5	9	1	0
54	4	4	12	10	14	2	2
50	0	0	0	0	2	0	0
48	0	0	0	3	3	2	2
40	4	1	4	0	6	0	1
34	1	8	15	2	10	0	0
30	2	0	1	0	4	0	0
29	0	0	0	1	1	2	2
26	0	0	1	0	0	2	0
24	1	1	3	0	0	0	0

Table 3: Table of gene homologies

VRE ABC 1

Homology	Identity Observed	Fasta Score	Reference
VRE Ribosomal sequence			
B.stearothermophilus L17	66% in 63 residues PO7843	200	(29)
B.subtilis L17	66% in 63 residues P20277	202	(6)
B.halodurans rplQ/L17	63% in 63 residues O50635	196	(52)
M.capricolum L17	51% in 60 residues PIR S48596	147	(5)
VRE ABC 1			
YbxA (YbaD) *B. subtilis*	57% in 271 residues P40735	818	(6, 40, 50, 55)
StrpA *Streptococcus pyogenes*	56% in 272 residues AF082738	802	(3)
YbxA homologue *B. halodurans*	48% in 273 residues AB017508	698	(52)
Y179 *Mycoplasma genitalium*	42% in 264 residues P47425 Haemolysin secretion	590	(19)
Protein O mth 133 *Methanobacterium thermoautotrophicum*	42% in 263 residues AE000928 Cobalt transport	521	(17, 48)
CbiO MJ1088 *Methanococcus jannaschii*	41% in 259 residues U67551 Cobalt transport	506	(7)
CbiO OT3 *Pyrococcus horikoshii*	40% in 281 residues PIR G64435 Cobalt transport	501	(28)
YkoD *B. subtilis*	30% in 249 residues AJ002571	436	(6, 40, 50, 55)
VRE ABC 2			
Yba E *B. subtilis*	72% in 162 residues P70970	749	(6, 40, 50, 55)
Cbi O *Propionibacterium freudenreichii*	45% in 161 residues U13043 Cobalt transport	317	(44)
Z3339 *Mycoplasma capricolum*	61% in 100 residues P14788 Sulphate transport	311	(5)
Art P *E.coli*	42% in 158 residues CAA 60101 Arginine transport	273	(4)
YkoD *B.subtilis*	40% over 168 residues AJ002571	255	(6, 40, 50, 55)
CbiO *Salmonella typhimurium*	40% in 168 residues L12006 Cobalt transport	253	(43)

Table 4: Results of in vivo assessment of the human recombinant antibodies

Expt No	Antibody Type (DNA Fingerprint)	Antibody Specificity	No. of mice	Organ Colony counts (mean $\log_{10}$ CFU/g ± SD)		
				Kidney	Liver	Spleen
1	Negative control		5	6.43 ± 1.33	6.74 ± 1.83	6.62 ± 1.58
	Type 1	Peptide 2	5	6.54 ± 1.31	6.42 ± 1.25	5.84 ± 0.72[b]
	Type 2	Peptide 2	5	6.55 ± 1.35	6.44 ± 1.54	6.31 ± 1.12
	Type 3	Peptide 2	5	6.65 ± 1.30	6.70 ± 1.14	5.91 ± 0.87[b]
2	Negative control		5	6.70 ± 1.12	6.68 ± 1.59	6.22 ± 1.11
	Type 1	Peptide 2	5	6.51 ± 1.49	6.57 ±1.32	5.62 ± 0.46[b]
	Type 2	Peptide 2	5	6.57 ± 1.56	6.23 ± 1.02[b]	5.64 ± 0.78[b]
	Type 3	Peptide 3	5	6.69 ± 1.15	6.66 ± 1.45	5.77 ± 0.30[b]
3	Negative control		10	5.72 ± 0.78	6.30 ± 1.42	4.66 ± 0.82
	Type 4	Peptide 2	10	5.20 ± 0.98[b]	5.71 ± 0.69[b]	4.72 ± 0.89
	Type 5	Peptide 2	10	5.16 ± 1.08[b]	5.96 ± 0.94	5.01 ± 1.11
	Type 6	Peptide 2	10	4.94 ± 0.99[b]	5.04 ± 1.26[c]	3.78 ± 0.85[b]
	Type 7	Peptide 3	10	5.66 ± 1.67	6.04 ± 1.03	4.78 ± 0.88
	Type 15	ABC2	10	4.70 ± 0.80[c]	5.30 ± 1.23[c]	4.08 ± 1.08[b]
4	Negative control		10	4.96 ± 1.07	5.57 ± 0.59	5.04 ± 1.08
	Type 6	Peptide 2	10	4.16 ± 0.37[b]	4.99 ± 0.86[b]	4.04 ± 0.95[c]
	Type 12	ABC 1	10	4.46 ± 0.81[b]	5.00 ± 1.17[b]	4.53 ± 1.67[b]
	Type 13	ABC 1	10	4.94 ± 1.09	4.94 ± 0.92[b]	4.36 ± 0.98[b]
	Type 15	ABC 2	10	4.50 ± 0.41	5.01 ± 0.94[b]	4.14 ± 0.96[b]
	Type 16	ABC 2	10	5.26 ± 1.37	5.49 ± 1.34	4.67 ± 0.79
5	Negative control		10	5.84 ± 1.83	5.38 ± 1.39	4.41 ± 1.32
	Type 6	Peptide 2	10	4.44 ± 0.82[c]	4.08 ± 1.16[c]	3.24 ± 0.29[c]
	Type 13	ABC 1	10	5.15 ± 1.26[b]	4.88 ± 0.82[b]	4.41 ± 0.66
	Type 14	ABC 1	10	5.53 ± 1.72[b]	5.20 ± 1.28	3.36 ± 0.30[c]
	Type 15	ABC 2	10	5.04 ± 1.04[b]	4.73 ± 0.64[b]	3.73 ± 0.69[b]
6	Negative control		10	5.82 ± 1.88	6.11 ± 1.08	5.30 ± 1.56
	Type 6	Peptide 2	10	5.07 ± 1.07[b]	5.08 ± 0.96[c]	4.22 ± 1.12[c]
	Type 8	Peptide 4	10	5.43 ± 1.62	5.55 ± 1.59[b]	4.51 ± 1.54
	Type 9	Peptide 4	10	4.99 ± 1.02[b]	5.41 ± 0.21	5.15[b] ± 1.36
	Type 10	Peptide 4	10	4.78 ± 0.87[c]	5.27 ± 1.19[b]	4.10[c] ± 1.06
	Type 11	Peptide 4	10	5.57 ± 1.73	5.45[b] ± 1.31	4.43[b] ± 1.56

a The phage dose was 10^8 ± 0.5 PFU. Mice were culled at 24h in experiment 1 but at 48h for all following experiments.

b Semilogarithmic reduction compared to negative control.

c Logarithmic reduction compared to negative control.

Figure 1: Immunoblot of VRE against: the peritoneal dialysate of an infected patient (Lane 1); after affinity selection (Lane 2); rabbit pre - (Lane 3) and post – (Lane 4) immunization with peptide 1; and rabbit pre – (Lane 5) and post – (Lanes 6 and 7) immunization with peptide 2.

Figure 2: **Immunoblots of VRE with paired sera from three patients who recovered form septicemia, showing the IgG present early in the infection (Lanes 1, 3 and 5 representing the three respective patients) and later in the infection (Lanes 2, 4 and 6 respectively corresponding to the same patients). Lane 7 is the antibody used for screening the VRE library. Molecular masses are marked in kDa.**

Figure 3

```
DNA: TGAAAATTTTGTCTTTTTTTGATAGTGTTAGTAGGAACTTTAGGATGAAGA    51

       RBS        VRE ABC1 ──►
DNA: AGGAGTAATCATGGAGCCAATTATTGAATTAGAAAAGATAAATTATAAATA   102
ABC1:            M  E  P  I  I  E  L  E  K  I  N  Y  K  Y    14
YbxA:     M  N  Q  N  Q  L  I  S  V  E  D  I  V  F  R  Y    16

DNA: CCAGCCAGATGATCTTCGCCCCGCATTGAAAGATGTCTCCTTTACAATTGA   153
ABC1:  Q  P  D  D  L  R  P  A  L  K  D  V  S  F  T  I  D    31
YbxA:  R  K  D  A  E  R  R  A  L  D  G  V  S  L  Q  V  Y    33

                                    Walker site A
DNA: TAAAGGTGAGTGGATCGCTATTATTGGACACAATGGTTCAGGAAAATCGAC   204
ABC1:  K  G  E  W  I  A  I  I  G  H  N  G  S  G  K  S  T    48
YbxA:  E  G  E  W  L  A  I  V  G  H  N  G  S  G  K  S  T    50

DNA: ACTTGCCAAAACGATTCAATGGATTGCTTCTGCCAGAATCAGGGATCGTGA   255
ABC1:  L  A  K  T  I  N  G  L  L  L  P  E  S  G  I  V  K    65
YbxA:  L  A  R  A  L  N  G  L  I  L  P  E  S  G  D  I  E    67

DNA: AAGTGGGAAATCAAATATTGGATGAAGAAAATATTTGGACGATCCGACAGA   306
ABC1:     V  G  N  Q  I  L  D  E  E  N  I  W  T  I  R  Q  M    82
YbxA:     V  A  G  I  Q  L  T  E  E  S  V  W  E  V  R  K  K    84

DNA: TGGTTGGAATGGTTTTTCAAAATCCAGACAATCAGTTTGTTGGTTCGACAG   357
ABC1:     V  G  M  V  F  Q  N  P  D  N  Q  F  V  G  S  T  V    99
YbxA:     I  G  M  V  F  Q  N  P  D  N  Q  F  F  G  T  T  V   101

DNA: TAGAAGATGACGTTGCTTTTGGTCTTGAAAATCAAGGGATTCCCAGAGAAG   408
ABC1:     E  D  D  V  A  F  G  L  E  N  Q  G  I  P  R  E  E   116
YbxA:     R  D  D  V  A  F  G  L  E  N  N  G  V  P  R  E  E   118

DNA: AAATGCTAGTGCGTGTGAAAGATGCACTTGAGAAAGTTAGAATGGCTGAGT   459
ABC1:     M  L  V  R  V  K  D  A  L  E  K  V  R  M  A  E  F   133
YbxA:     M  I  E  R  V  D  W  A  V  K  Q  V  N  M  Q  D  F   135

                              Linker peptide
DNA: TTGCTTCACGCGAGCCTGCTCGTTTATCAGGAGGGCAAAAACAACGTGTTG   510
ABC1:     A  S  R  E  P  A  R  L  S  G  G  Q  K  Q  R  V  A   150
YbxA:     L  D  Q  E  P  H  H  L  S  G  G  Q  K  Q  R  V  A   152

                                    Walker site B
```

```
DNA:  CCATTGCCGGCGTGGTTGCCTTACGACCTGATATCATTATTTTAGATGAAG   561
ABC1:   I  A  G  V  V  A  L  R  P  D  I  I  I  L  D  E  A   167
YbxA:   I  A  G  V  I  A  A  R  P  D  I  I  I  L  D  E  A   169

DNA:  CAACCAGTATGCTAGATCCAGAAGGTCGTGAAGAAGTGATCTCTACGATCA   612
ABC1:   T  S  M  L  D  P  E  G  R  E  E  V  I  S  T  I  K   184
YbxA:   T  S  M  L  D  P  I  G  R  E  E  V  L  E  T  V  R   186

                                    Switch region
DNA:  AGAAGATCAAAGAAGAAAGTCAATTGACCGTTATATCTATCACTCATGATA   663
ABC1:   K  I  K  E  E  S  Q  L  T  V  I  S  I  T  H  D  I   201
YbxA:   H  L  K  E  Q  G  M  A  T  V  I  S  I  T  H  D  L   203

DNA:  TTGATGAAGCAGCTAATGCCAATCGGATACTAGTGATGAGACAAGGCGAAC   714
ABC1:   D  E  A  A  N  A  N  R  I  L  V  M  R  Q  G  E  L   218
YbxA:   N  E  A  A  K  A  D  R  I  I  V  M  N  G  G  K  K   220

DNA:  TTGTCCGTGAAGGTACACCAAAAGAAATTTTTTCTGCTGGTCCTGAACTGA   765
ABC1:   V  R  E  G  T  P  K  E  I  F  S  A  G  P  E  L  I   235
YbxA:   Y  A  E  G  P  P  E  E  I  F  K  L  N  K  G  L  V   237

DNA:  TCGACTTAGGTCTAGATCTACCATTTCCTGAAAAACTGAAGAGCGCACTGA   816
ABC1:   D  L  G  L  D  L  P  F  P  E  K  L  K  S  A  L  K   252
YbxA:   R  I  G  L  D  L  P  F  S  F  Q  L  S  Q  L  L  R   254

                                                      RBS
DNA:  AAGAACGTGGGGTTGATGTGCCCAGTGAATATATGACAGAAGAAAGGATGG   867
ABC1:   E  R  G  V  D  V  P  S  E  Y  M  T  E  E  R  M  V   269
YbxA:   E  N  G  L  A  L  E  E  N  H  L  T  Q  E  G  L  V   271

                VRE ABC2  ⟶
DNA:  TGGATTGGTTATGGACATCCGTTTTGAACAAGTAGACTTCACTTATCAGCC   918
ABC2:              M  D  I  R  F  E  Q  V  D  F  T  Y  Q  P    14
YbaE:                                                   M    1
ABC1:   D  W  L  W  T  S  V  L  N  K  *                     279
YbxA:   K  E  L  W  T  L  Q  L  K  M  *                     281

DNA:  GAATACACCATTTGAACAAAGAGCCTTATTTGACATCAATATGACGATCAA   969
ABC2:   N  T  P  F  E  Q  R  A  L  F  D  I  N  M  T  I  K    31
YbaE:   K  T  P  F  E  R  L  A  L  Y  D  I  N  A  S  I  K    33

                                    Walker site A
DNA:  AGAAAACAGTTATACTGCATTAGTAGGGCATACCGGAAGCGGGAAATCAAC  1020
ABC2:   E  N  S  Y  T  A  L  V  G  H  T  G  S  G  K  S  T    48
YbaE:   E  G  S  Y  V  A  V  I  G  H  T  G  S  G  K  S  T    50

DNA:  CTTACTTCAGCATTTGAATGCGCTTGTTAAGCCAACAAGCGGTACAGTCCA  1171
ABC2:   L  L  Q  H  L  N  A  L  V  K  P  T  S  G  T  V  H    65
YbaE:   L  L  Q  H  L  N  G  L  L  K  P  T  K  G  Q  I  S    67
```

```
 DNA: TATTGGAGAACGAGATATTCAGCCGGATACGGATAATAAGAATTTAAAGCC   1222
ABC2:  I  G  E  R  D  I  Q  P  D  T  D  N  K  N  L  K  P    82
YbaE:  L  G  S  T  V  I  Q  A  G  K  K  N  K  D  L  K  K    84

 DNA: CATCCGAAAAAAAGTTGGCATCGTCTTTCAGTTTCCAGAAGCACAGCTATT   1273
ABC2:  I  R  K  K  V  G  I  V  F  Q  F  P  E  A  Q  L  F    99
YbaE:  L  R  K  K  V  G  I  V  F  Q  F  P  E  H  Q  L  F   101

 DNA: CGAAGAAACGGTAGCAAAAGACATTGCTTTCGGTCCTAAAAACTTTGGTGT   1324
ABC2:  E  E  T  V  A  K  D  I  A  F  G  P  K  N  F  G  V   116
YbaE:  E  E  T  V  L  K  D  I  S  F  G  P  M  N  F  G  V   118

 DNA: CAGCGAAGAAGAAGCACTAGTCCTAGCAAAAGAAACATTAGAACAAGTTGG   1375
ABC2:  S  E  E  E  A  L  V  L  A  K  E  T  L  E  Q  V  G   133
YbaE:  K  K  E  D  A  E  Q  K  A  R  E  M  L  Q  L  V  G   135

                                          Linker peptide
 DNA: GCTGGATGAAAGCTATTTGGAACGTTCGCCATTTGAACTTTCAGGAGGGCA   1426
ABC2:  L  D  E  S  Y  L  E  R  S  P  F  E  L  S  G  G  Q   150
YbaE:  L  S  E  E  L  L  D  R  S  P  F  E  L  S  G  G  Q   152

 DNA: AATGCGTAGAGTAGCAATCGCTGGTGTGCTTGCCATGAGGCCGGAAGTACT   1477
ABC2:  M  R  R  V  A  I  A  G  V  L  A  M  R  P  E  V  L   167
YbaE:  M  R  R  V  A  I  A  G  V  L  A  M  D  P  E  V  L   169

       Walker site B
 DNA: TGTATTGGATGAACCAACGGCAGGACTTGAT                       1508
ABC2:  V  L  D  E  P  T  A  G  L  D Vector                 177
YbaE:  V  L  D  E  P  T  A  G  L  D cont..                 179
```

Legend
RBS = Ribosomal binding site

Figure 3: Enterococcal ABC transporter DNA and amino acid sequences. The *YbxA* and *YbaE* protein amino acid sequences are shown underneath for comparison. Epitopes in italics.

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Demonstration of activity of purified antibody

Antibody fragment 1.1 against peptide LKPIRK KVGIVEQTP (sequd 6) was amplified using PCR with primers:

GTCCTCGCAACTGCGCATATGGCCCAGGTGCAGCTGGTGCAGTCTGGG

and CGATCTAAAGTTTTGTCGTCTTTCC

and subcloned into the pCR 2.1-TOPO TA cloning vector (Invitrogen). The plasmid was isolated using wizard plus SV minipreps (Promega) and fragment 1.1 digested from the vector using Nde1 and Not1 restriction enzymes. The resulting fragment was ligated into pET 29a plasmid (Novagen) and transformed into JM109 (De3) *E. coli*. Positive clones were grown in 6 x 0.5L LB broth, expression was induced by the addition of 0.1M IPTG, after 3 hours the cells were centrifuged at 4000 x g for 30 minutes. The cells were disrupted using a french press and the inclusion bodies washed 5 times in Lysis buffer (100mM KCl; 50mM Tris HCI PH8; 1mM EDTA; 2mM Deoxycholic acid), the protein was refolded over 3 days in refolding solution (2% N-lauryl Sarcosine; 50mM Tris pH9). The protein was diafiltered against 40 volumes of 10mM Ammonium acetate (pH9) and His tagged scfv purified using IMAC superflow resin (Quiagen). The resulting purified recombinant protein was dialysed overnight in formulation buffer (0.5M Urea; 100mM Arginine) and stored at –20°C. Purified antibody fragment was used in subsequent experiments at a dose of 25,50 or 75 μg.

Assessment in an animal model

Vancomycin resistant *Enterococcus faecium* isolates were obtained from the ATCC *E. faecium* ATCC 700221 (Strain 1) and from a clinical infection (Strain 2) (minimum inhibitory concentration ≥ 250 μg/ml). A vancomycin sensitive strain was also obtained from a patient (Strain 3).

All three strains were grown overnight in brain heart infusion at 37°C and washed in saline, and the concentration was determined by hemocytometer and by plating of dilutions on blood agar.

Next either 10^8 or 10^9 colony forming units were injected as a 0.1 ml bolus into the lateral tail vein of 22 to 24 g female CDI mice (Charles River). Two hours after inoculation, randomized groups of animals were given intravenously 100 µl of formulation buffer containing 0, 25, 50, or 75 µg of purified antibody fragment.

Animals were culled at 48 hours and bacterial cell counts were made from kidney, liver and spleen and expressed as the mean $\log_{10}$ cfu per gram plus standard deviation.

Results

Experiments 1-3 showed a reduction of logarithm 0-73-1.19 (kidney), 0.75-1.6 (liver) and 1.02-1.18 (spleen), in counts dependent on inoculum and dose of antibody fragment for Strain 1.

In experiment 4, Strain 2 failed to establish an infection in the kidney. In the spleen antibody therapy led to the majority becoming sterile and there was an 0.85 logarithm reduction in the liver count. Strain 3 at an inoculum of 10^8 failed to establish infection in the kidney and at 10^9 led to a colony count of 4.54 which was abolished by antibody. The mean logarithm drops for the liver were 1.6 and 1.82 and for the spleen there were 0.73 and 1.2 dependent on the inoculum.

Overall this demonstrated the activity of the pure antibody fragment against two strains of VRE and a vancomycin sensitive strain of *E. faecium*.

Expt No:	Antibody	Inoculum	Isolate	No. of mice	Colony counts (mean $\log_{10}$ (cfu/g ±SD)		
					[a]Kidney	[a]Liver	[a]Spleen
1.	0µg	10^8	Strain 1	15	5.23±0.24	6.57±0.31	5.87±0.76
	75µg	10^8	Strain 1	15	4.50±0.27	5.53±0.76	4.82±0.85
2.	0µg	10^9	Strain 1	10	5.80±0.97	6.86±1.01	5.74±0.44
	25µg	10^9	Strain 1	10	4.62±0.51	5.83±0.75	4.78±0.59
	50µg	10^9	Strain 1	10	4.61±1.1	5.26±1.18	4.7±0.58
3.	0µg	10^9	Strain 1	10	5.85±0.82	6.83±0.72	5.80±0.7
	50µg	10^9	Strain 1	10	4.94±0.77	6.26±0.84	4.91±0.57
	75µg	10^9	Strain 1	10	4.67±0.48	6.08±0.65	4.62±0.44
4.	0µg	10^8	Strain 2	10	- (8)	5.37±1.29	4.12±1.28(3)
	25µg	10^8	Strain 2	10	-(10)	4.79±1.98(2)	-(8)
	50µg	10^8	Strain 2	10	-(10)	4.52±1.45(2)	-(8)
5.	0µg	10^8	Strain 3	10	-(10)	5.44±0.45	4.26±1.16
	25µg	10^8	Strain 3	10	-(10)	5.41±0.72	4.1±0.97
	50µg	10^8	Strain 3	10	-(10)	3.84±0.68	3.53±0.33(3)
6.	0µg	10^9	Strain 3	10	4.54±1.37(5)	6.32±0.79	4.85±1.0
	75µg	10^9	Strain 3	10	-(12)	4.5±0.32	3.65±0.45

[a] In brackets number of sterile organs. When greater than 50% no count is given.

Table 1